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13. ABSTRACT (Maximum 200 Words) The overall goals of this project are to 1) identify candidate clock genes by analyzing the spatial expression patterns and circadian activity rhythm phenotypes of enhancer detector P-element insertion lines and 2) isolate these genes (or cDNA copies thereof) using the P-element as a molecular marker. We have screened through >3000 independent insertion lines. Among these, ~2% of the lines produced spatial expression patterns similar to that of known clock genes, <i>period</i> and <i>timeless</i> . Behavioral rhythms were tested in 63 lines, and only one line consistently gives a ~1 hour lengthening in period. The insert was characterized and found to reside in <i>period</i> gene regulatory sequences. We have used this line to define the developmental expression of the <i>period</i> gene, identify cell types during larval and pupal development, and to study <i>period</i> gene regulation by creating mutations due to imprecise excision of this insertion. By screening through 150 additional insertion lines for behavioral alterations, we have identified 2 lines which are arrhythmic. These lines are currently being verified by reverting the phenotypes through precise excision of the inserts. These studies have provided insight into <i>period</i> gene expression at all developmental stages and may ultimately result in the isolation of new clock genes.					
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INTRODUCTION

To understand how the circadian system operates to effect temporal order, the time-keeping mechanism (pacemaker) and its various outputs to overt rhythms must be defined. A breakthrough in this regard has come from genetic screens for mutants that affect circadian behavior. The best characterized rhythm mutants in *Drosophila* are within the period (*per*) gene (15), which has been crucial for determining that a molecular feedback loop is part of the molecular mechanism governing pacemaker function (5, 8, 11, 31) and that a set of neurons in the lateral protocerebrum (LNs) are important for behavioral rhythmicity (7, 9). How this feedback loop functions to keep time or activate behavioral outputs is largely unknown. To better define the mechanisms involved in regulating the *per* feedback loop and its behavioral output we have used an enhancer detector screening strategy to identify new clock genes.

The levels of *per* RNA and protein cycle in a circadian manner, where *per* mRNA peak (occurring at ~ZT15) phase leads the *per* protein peak (occurring at ~ZT21) by approximately 6hr (6, 11, 26, 31). These fluctuations are seen during 12hr. light:12hr. dark cycles (LD) and persist under free-running (in constant darkness or DD) conditions, indicating that these are true molecular circadian rhythms (6, 11, 31). The *per* mutants influence the phase (in LD) and period (in DD) of *per* RNA and protein cycling in parallel to their effects on behavioral rhythms (6, 11, 31). These results suggest that *per* molecular oscillations constitute a feedback loop whereby *per* mRNA is the template for PER synthesis and PER is necessary for circadian fluctuations in *per* RNA (11, 12). Another gene that contribute to this feedback loop is timeless (*tim*), which is rhythmically expressed in phase with *per* (25). The *tim* gene product (TIM) is required for PER to enter the nucleus, where it feeds back to regulate its own (and probably *tim*) transcription (29). The *per* gene is expressed in many different neuronal and non-neuronal tissues in the head (i.e. photoreceptors, antennae, brain glia, LNs, dorsal neurons (DNs) proboscis) and the body (i.e. cardia, thoracic ganglion, gut, ovaries, testes) (7, 17, 23, 26). The circadian feedback loop appears to be operating in all of these tissues except the ovary (10), but the only "tissue" connected to an output are LNs.

Since PER expression in LNs is sufficient to mediate circadian activity, we would like to know what genes other than *per* (and presumably *tim*) are involved in this process. In addition, we would like to identify other genes that are involved in regulating the molecular cycling within the *per* feedback loop. Classically, screens for genes involved in mediating circadian activity have centered on uncovering mutants having altered eclosion or locomotor activity (13, 15, 16, 20, 24). However, these screens have several disadvantages which make them laborious, time consuming and troublesome: 1) The genetic background of the stocks used for mutagenesis must be extensively tested behaviorally so as to minimize period variability, 2) Monitoring eclosion or locomotor activity rhythms takes one to two weeks and requires several individuals to ensure reproducibility, 3) The cost of behavioral monitoring equipment is prohibitive (~\$1000 to measure 32 individuals), 4) Putative mutants must be outcrossed and genetically mapped to ensure that they are due to single mutations. To identify genes that control locomotor activity rhythms or are part of the circadian feedback loop, we will screen for genes specifically expressed in LNs or all *per* expressing cells. Genes expressed in these patterns will be identified using "enhancer detector" transposons (18).

Genes expressed in a spatially restricted pattern are generally regulated at the transcriptional level by enhancer elements (18). To search for enhancers in the *Drosophila* genome, we have used a P-element vector having the following features: 1) A "basal" promoter which is only active when situated near an enhancer, 2) The *E. coli* lacZ gene is driven by the basal promoter so that expression patterns can be easily detected by X-gal staining, 3) An easily scorable white eye color marker (2, 18, 21, 30). Enhancer detector vectors introduced into the genome via germline transformation are mobilized to generate insertions throughout the genome using a source of P-element transposase that is stably integrated into the genome (3, 22). Specific expression patterns of each single insertion strain are then assayed by X-gal staining. In several cases that have been directly examined, the pattern of lacZ expression mirrors the expression pattern of a gene situated near the insertion site (1, 2, 21, 30).

When the promoter driving lacZ gene expression responds to the same enhancer that drives

an endogenous genes' expression, the expression of that gene is not necessarily disrupted (18). In accord with this, only ~10% of P-element insertion sites lead to lethality (3). Presumably a higher percentage of insertion sites have little or no affect on phenotypes that are easy to score (i.e. viability, sterility, morphology or color phenotypes) or have variable affects on phenotypes that are either not obvious or are difficult to measure (i.e. behavioral phenotypes). If an enhancer trap insert is being expressed in the tissue of interest but does not produce a mutant phenotype, excision of the P-element, which often occurs imprecisely, may cause deletions in flanking genes which then result in a mutant phenotype (18). Such excision analysis can verify that the gene marked by the enhancer trap insert is involved in the phenomenon being studied.

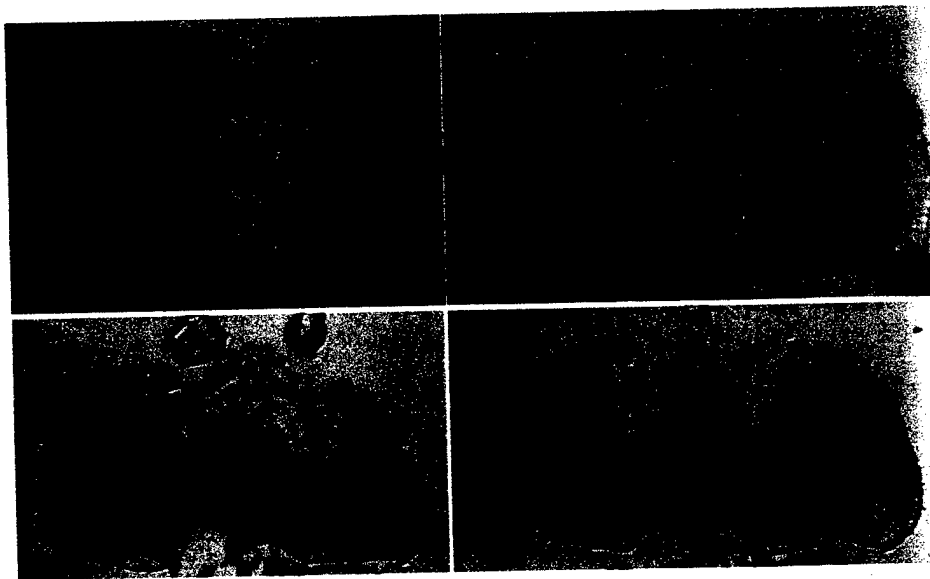
Enhancer trap screening overcomes many of the difficulties associated with conventional behavioral screening of chemically mutagenized flies. Specific advantages that enhancer trap screening provides over behavioral screening strategies are: 1) Assays for lacZ expression can be done in flies heterozygous for the enhancer trap insert, thereby reducing the amount of time, effort and supplies involved in homozygosing inserts, 2) The pattern of expression from enhancer trap inserts are not subject to variation due to genetic background which confounds many behavioral screens, 3) LacZ expression assays take one day to do rather than days to weeks for many behavioral assays, 4) Enhancer trap expression can be reliably assayed from single flies, which increases throughput compared to behavioral assays where many flies must be measured to derive a reliable phenotype 5) The enhancer that has been trapped is necessarily close to the P-element insert, while chemically mutagenized flies provide no molecular tag at all, and P-element generated mutants must be mapped genetically to ensure they map near the mutation, and 6) Mutants can be generated by imprecise excision of P-elements if the initial insert does not induce a mutation.

RESULTS & DISCUSSION

I. Isolation of enhancer trap lines that are expressed in a lateral neuron containing subset of the *per* expression pattern.

In this project we have screened over 3000 enhancer detector lines for spatial expression patterns.

This was accomplished using two strategies: jumping the enhancer detector from the X chromosome to create autosomal jumps and jumping the enhancer detector from a second chromosome balancer to create inserts on any chromosome. From these screens, 62 lines have been obtained



which express lacZ either in cells corresponding to LNs, or throughout the *per* expression pattern (Fig. 1). These lines are considered "positively" staining lines.

II. Analysis of enhancer trap lines expressed in an LN containing subset of the PER expression pattern.

Table 1. Behavioral rhythms of inserts exhibiting PER-like spatial expression.

Genotype (temp)	Rhythmic (total)	period +/- sem	Genotype (temp)	Rhythmic (total)	period +/- sem
wild type	15 (15)	24.3 +/- 0.10	7236-4	8 (10)	24.0 +/- 0.08
gg8	44 (50)	25.5 +/- 0.08	10236-76	10 (13)	24.0 +/- 0.18
kk39	9 (9)	24.0 +/- 0.06	10236-64	10 (11)	24.1 +/- 0.11
a10	10 (10)	23.8 +/- 0.07	10236-74	10 (12)	23.8 +/- 0.18
ee44	5 (9)	23.4 +/- 0.08	10236-66	11 (14)	23.8 +/- 0.08
r30	7 (7)	23.8 +/- 0.12	10236-77	23 (27)	24.0 +/- 0.09
aa3	4 (5)	23.9 +/- 0.06	7316-1	10 (10)	24.1 +/- 0.10
ii3	5 (5)	23.7 +/- 0.08	7316-4	7 (8)	23.8 +/- 0.20
rr38	5 (5)	23.8 +/- 0.07	8056-2	8 (8)	24.2 +/- 0.13
b11	4 (4)	23.9 +/- 0.09	7056-2	5 (6)	24.2 +/- 0.17
a15	6 (6)	23.8 +/- 0.07	9116-1	4 (5)	23.8 +/- 0.17
e4	6 (6)	23.8 +/- 0.1	9116-2	5 (5)	23.6 +/- 0.17
ii8	6 (6)	23.8 +/- 0.08	8056-2	2 (6)	24.6 +/- 0.45
h4	5 (5)	23.7 +/- 0.13	8056-13	5 (8)	23.8 +/- 0.19
h3	6 (6)	23.9 +/- 0.06	8086-13	7 (8)	23.6 +/- 0.15
pp4	5 (9)	23.9 +/- 0.12	8086-1	6 (7)	24.1 +/- 0.30
oo1	8 (11)	24.2 +/- 0.15	8096-18	7 (8)	24.0 +/- 0.22
pp8	7 (8)	24.0 +/- 0.16	9136-5	9 (9)	23.6 +/- 0.12
qq9	11 (12)	24.0 +/- 0.07	9136-2	4 (6)	23.7 +/- 0.07
7056-8	10 (13)	24.4 +/- 0.16	9136-1	5 (7)	23.8 +/- 0.15
7126-2	10 (12)	23.9 +/- 0.27	9116-7	7 (7)	23.8 +/- 0.14
7126-4	11 (13)	24.3 +/- 0.15	9116-9	10 (10)	23.6 +/- 0.12
7126-10	9 (14)	24.1 +/- 0.20	9116-8	8 (8)	23.9 +/- 0.14
7236-5	7 (15)	24.5 +/- 0.20	8096-6	6 (7)	24.2 +/- 0.09
7296-2	6 (9)	23.9 +/- 0.13	7103-3	9 (9)	23.9 +/- 0.17
10016-2	10 (10)	24.2 +/- 0.20	7027-1	9 (9)	24.3 +/- 0.14
7107-5	7 (7)	23.8 +/- 0.16	7217-1	7 (7)	24.0 +/- 0.06
7187-6	5 (7)	24.1 +/- 0.23	7257-2	9 (10)	23.9 +/- 0.11
7227-18	8 (8)	23.7 +/- 0.17	7237-1	9 (10)	23.6 +/- 0.11
7237-3	9 (9)	24.0 +/- 0.16	9247-6	8 (9)	23.7 +/- 0.09
121897-24	9 (10)	24.0 +/- 0.12	121898-25	8 (9)	23.6 +/- 0.13
121897-74	9 (10)	23.7 +/- 0.10			

Flies were entrained for three days in 12 h light: 12 h dark cycles and monitored for seven days in constant darkness at 25°C. Period values were determined by X² periodogram analysis. Flies were scored as rhythmic if they had powers (height of the most significant period value above the 5% significance line) of >10 and widths (number of consecutive period values above the 5% significance line) of >2.

a) Monitoring behavioral rhythms of positive lines.

The 63 lines expressed in an LN containing subset of the PER expression pattern were tested for locomotor activity rhythms using the Trikinetics Drosophila Activity Monitoring (DAM) system. Monitoring was done for 7 days in constant dark (DD) after 3 days of entrainment in 12hr light:12hr dark (LD) cycles. In each line the proportion of rhythmic flies was close to that of wild type (CS) flies (Table 1). Only the *gg8* line produced behavioral rhythms significantly different in period than that of CS. 44 *gg8* flies were tested for behavioral activity and we find that their average free-running period is ~25.5 h compared to wild type ($\tau \sim 24.2$ h) or the other P-element insert lines (Table 1).

b) Behavioral analysis of *gg8*.

The *gg8* insert mapped to the multiply inverted second chromosome balancer (used to suppress recombination) that is itself homozygous lethal. Thus, we inferred that *gg8* was producing this phenotype as a heterozygote. Since *tim* is on the second chromosome, we wanted to determine whether *gg8* was an allele of *tim*. Table 2 shows that *gg8* is not a *tim* allele because we would expect that the period would be even longer than *gg8/+*, but the period is that of wild type. Next we sought to determine whether the phenotype was due to the insert rather than a second site mutation. When the insert was excised, the phenotype reverted back to that of wild type flies (Table 2), consistent with the mutation mapping to the insert.

Given these data, we were optimistic that the *gg8* enhancer detector insertion marks a new clock gene. However, when was outcrossed to produced *rosy+* (which contain the second chromosome insert) and *rosy* progeny (which lack the second chromosome insert), the *rosy* progeny also maintained *per/tim*-like expression and lengthened behavioral rhythms. This was very surprising considering the experiment was done as a control, but it clearly shows that the enhancer trap was not due to the second chromosome insert. Since the insert producing the enhancer trap pattern has lost its external phenotypic marker, mapping the insert was accomplished by staining for the enhancer trap pattern. Using this method, the insert was mapped to the X chromosome.

Table 2. Behavioral rhythms of a *gg8* revertant and *gg8/tim* flies.

Genotype	Rhythmic (total)	period \pm sem
<i>gg8/+</i>	22 (24)	25.7 \pm 0.10
<i>gg8rev1/+</i>	5 (5)	24.2 \pm 0.10
<i>tim/tim</i>	0 (17)	
<i>tim/+</i>	13 (13)	23.8 \pm 0.12
<i>gg8/tim</i>	20 (20)	23.9 \pm 0.12

Flies were entrained, tested and scored as in Table 1.

c) Molecular analysis of *gg8*.

Concurrent with these genetic experiments we used inverse PCR to isolate flanking sequences from the *gg8* insert. We cloned an 800bp DNA fragment flanking the insertion site and sequenced this insert. The sequence was essentially identical to that of *per*. Based on the sequence data, this insert landed approximately 200bp upstream of the *per* gene. This result was thoroughly confirmed via PCR of *gg8* genomic DNA using primers in the *per* upstream sequence and within the enhancer detector P-element (Fig. 2).

The finding that *gg8* was an enhancer trap of the *per* gene explains our molecular and behavioral results. An insert 200bp upstream of the *per* start site apparently trapped the *per* transcriptional regulatory apparatus, explaining the beautiful recapitulation of *per* spatial expression and the cycling of *lacZ* reporter gene transcript. An insert at this position is likely to disrupt

expression of the *per* gene, which would account for the altered behavioral rhythm. Even though this insert did not mark a new clock gene, it is significant in that it verifies that this approach is valid and can disrupt clock genes.

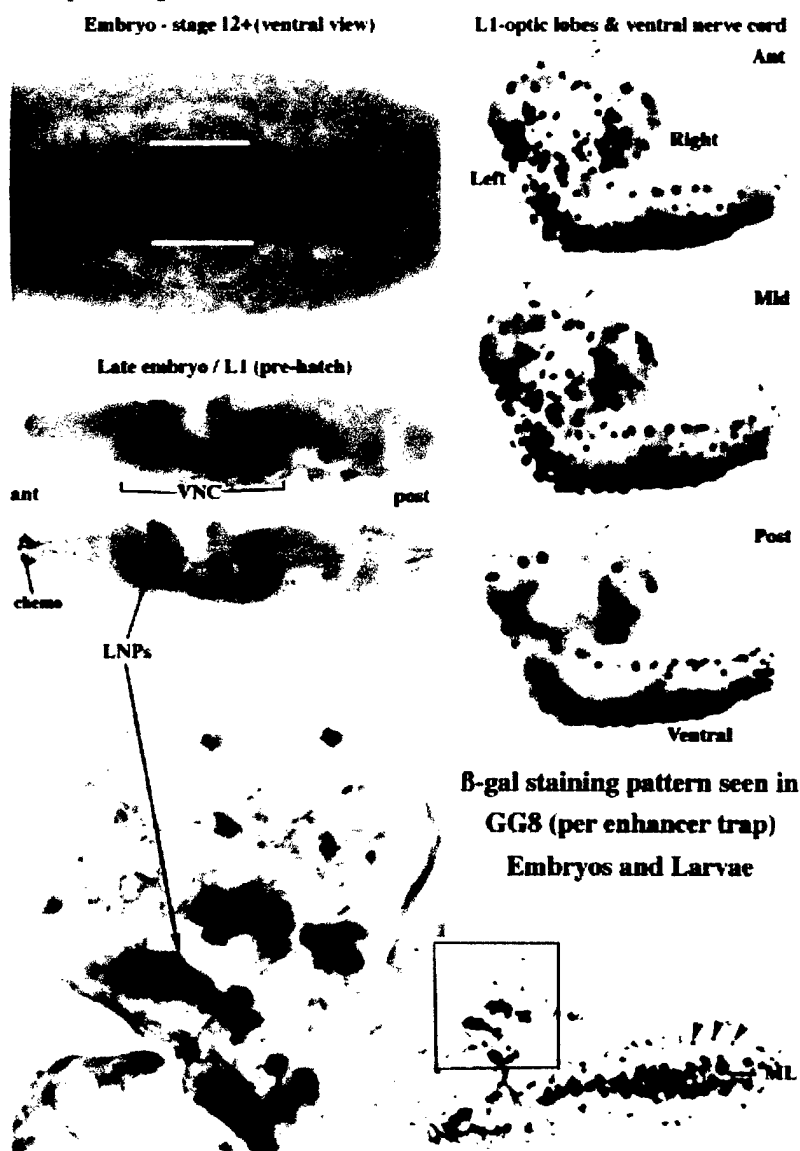
Since *gg8* is inserted just upstream of the *per* transcription start site and shows a ~1.5hr lengthening of the rhythm, we want to use this insert to generate larger lesions in the *per* promoter and determine what the behavioral effects will be. If lesions are large enough to eliminate *per* expression, the flies should be arrhythmic. We have generated >100 excision strains and tested 46 of them for activity rhythms. Of the 46, four show longer periods in the 26.5hr - 28hr range. We are using PCR to determine the extent of lesions created by the imprecise excision of the transposons. These studies will complement our analysis of regulatory elements within the *per* promoter.

d) Developmental analysis of *gg8*.

We have used the *gg8* line to define *per* expression at earlier stages of development. *per* is expressed in many cells in late embryos and larvae (Fig. 3). The *gg8* expression pattern is more extensive than the PER staining pattern in larvae (14), indicating that the protein never accumulates in some cells in which the *per* gene is expressed. This restriction in PER accumulation may be due to a lack in the expression of TIM, which acts to stabilize PER, or some other factor that effects post-transcriptional regulation. Post-transcriptional mechanisms have been invoked for regulating circadian mRNA and protein cycling (4, 19, 27, 28), but this is the first indication that spatial expression of PER may be limited by post-transcriptional mechanisms.

III. Behavioral analysis of random transposon insertion lines.

Since we have not identified any new clock genes from our enhancer trap screen, we tested other random transposon insertions to determine if they affected circadian behavior. These tests involved small numbers of homozygous inserts. We have tested 69 lines thus far and have found six that show an altered activity rhythm. In each case, a large proportion of the flies (>80%) are arrhythmic.



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